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Metabolic stress induced by arginine deprivation induces autophagy cell death in prostate cancer

PRINCIPAL INVESTIGATOR:
Richard Bold, M.D.

CONTRACTING ORGANIZATION:
University of California, Davis
Davis, CA 95618

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14. ABSTRACT The primary purpose of this research grant is to provide the necessary preclinical data demonstrating that prostate cancer cells are auxotrophic for arginine and therefore targeting arginine metabolism is a novel therapeutic approach. The primary methodology involves cell culture with the characterization of the arginine requirements for prostate cancer cell growth and then determination of the effect of arginine depletion on cell growth and cell death. Furthermore, we have investigated the mechanism of cell death and observed that arginine deprivation in those cells auxotrophic for this semi-essential amino acid induces autophagy as a precursor to programmed cell death. Major findings to date include the observation that the majority of prostate cancer cell lines lack arginine-succinate synthetase (ASS), the critical enzyme in arginine biosynthesis. Furthermore, arginine deprivation in those cell lines lacking ASS induces autophagy as a precursor to non-apoptotic cell death. Inhibition of autophagy appears to stimulate the induction of cell death.					
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Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4-6
Key Research Accomplishments.....	6
Reportable Outcomes.....	6-7
Conclusion.....	7
References.....	7
Appendices.....	8-16

Introduction

This is the first of a three year grant entitled “Metabolic stress induced by arginine deprivation induces autophagy cell death in prostate cancer”. The primary hypothesis of the research investigation is PEG-ADI represents a potential therapy of prostate cancer to induce metabolic stress by arginine deprivation and subsequent induction of autophagic cell death as a precursor to apoptosis in those prostate cancers lacking biosynthetic enzymes to make arginine. The specific aims to investigate this hypothesis are:

- 1) We will demonstrate that in those prostate cancer cells lacking ASS for which arginine is an essential amino acid, metabolic stress induced by arginine deprivation achieved by ADI-PEG treatment induces autophagic cell death as a precursor to apoptosis via a DRAM-dependent pathway. From these studies, we will show: 1) ASS expression predicts cellular response to PEG-ADI, 2) PEG-ADI therapy induces arginine deprivation, 3) the consequence of the metabolic stress is the initiation of autophagosome assembly and progression to autophagic cell death, and 4) DRAM induction is required for PEG-ADI induced autophagy.
- 2) We will demonstrate that the induction of PEG-ADI induced autophagy in prostate cancer cells is a precursor to apoptosis; furthermore, these events sensitize cells to traditional chemotherapy-induced apoptosis. Autophagy mediators will be altered to determine effect on sensitivity to PEG-ADI induced cellular events. In addition, PEG-ADI will be combined with traditional chemotherapy to determine effect on apoptosis and in vivo tumor response.

Body

Significant progress has been accomplished during the first year of this 3-year award leading to two publications (1, 2). The initial focus of the investigation was the characterization of the auxotrophic requirements for prostate cancer cell lines for the semi-essential amino acid arginine. We have used two methods of arginine deprivation, arginase and arginine deiminase (ADI). Both of these enzymes shuttle arginine away from the urea cycle and have been demonstrated to reduce intracellular arginine. Arginase has limited clinical applications due to rapid plasma degradation; we have been fortunate to develop a collaborative research agreement with Polaris Pharmaceuticals, who have developed a pegylated form of ADI (PEG-ADI) that has reduced serum degradation. A necessary requirement for the proposed studies was to demonstrate that intracellular arginine was in fact depleted following PEG-ADI treatment. We used HPLC to characterize amino acid levels in CWR22rV1 cells, which we had demonstrated lacks the enzyme argininosuccinate synthetase (ASS), the rate-limiting step in arginine metabolism. We noted that withing 24 hours of PEG-ADI treatment, arginine levels had decreased to undetectable levels (Figure 1).

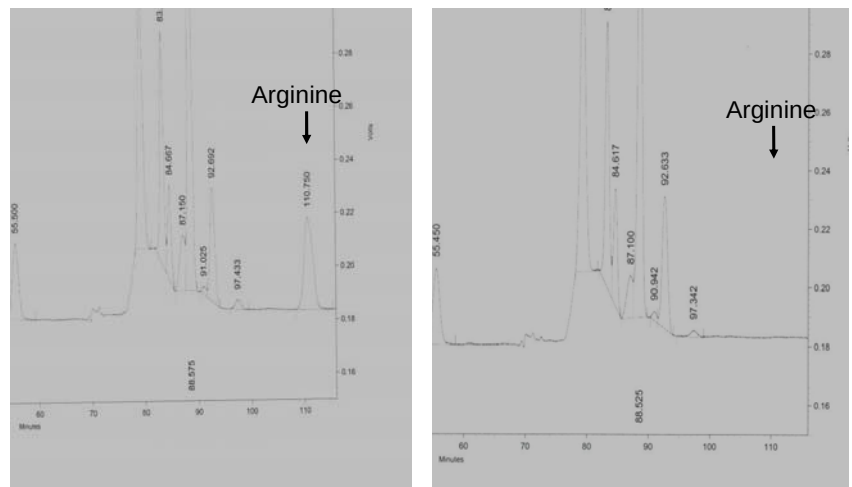


Figure 1. Levels of amino acids in exponentially growing CWR22 cells (left) and 24 hrs after treatment with PEG-ADI (1 µg/ml) (right)

We have since characterized the expression of ASS in a panel of human prostate cancer cell lines as well as a tissue microarray of human prostate tumor specimens (1). We noted that there was a frequent lack of expression of ASS. The cellular effect of arginine deprivation achieved by PEG-ADI treatment was then determined both in vitro and in vivo. We observed that cell lines deficient in ASS expression underwent autophagy and programmed cell death, though not the traditional caspase-

dependent apoptosis (1). Our data suggests that expression of ASS is a predictive marker of response to PEG-ADI, and only those prostate cancer cell lines auxotrophic for arginine will undergo programmed cell death following arginine deprivation (2). We have further investigated the role of autophagy in the cellular response of these prostate cancer cell lines. Our studies have primarily utilized CWR22rV1, which lacks ASS expression. Using both pharmacologic inhibitors of autophagy (chloroquine) as well as molecular inhibitors (siRNA-mediated knockdown of Beclin-1, a protein necessary for the initial assembly of the autophagosome), we noted that when autophagy is inhibited, the cell death is accelerated following PEG-ADI treatment (reference 1 manuscript, figure 5, attached in appendix). These data suggest that autophagy following PEG-ADI participates in the induction of cell death by providing a survival mechanism (2).

In order to demonstrate that lack of ASS expression is both required for the cell death following PEG-ADI treatment as well as useful as a predictive marker, we have cloned the ASS cDNA. In brief, we used the NIH-MGC-75 library to perform PCR for the ASS cDNA from the Image Clone ID No. 4042389 in the pDNR-LIB vector from the IRAU library. It has subsequently been subcloned into the eukaryotic expression vector, pCDNA3.1. We have initially performed transfection into the CWR22rV1 cell line, which lacks endogenous ASS expression (Figure 2). This cell line will now allow us to directly test our hypothesis that ASS expression correlates with cellular response to arginine deprivation achieved by PEG-ADI treatment.

ASS Level in CWR22RV1 After ASS cDNA Transfection

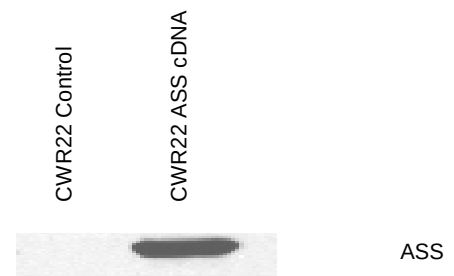
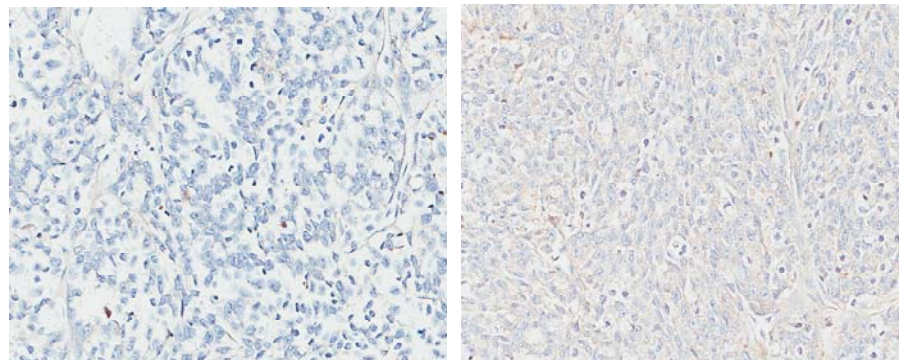


Figure 2. Evaluation of ASS expression in CWR22rV1 cells before and after transfection of a eukaryotic expression vector encoding the ASS cDNA. The CWR22rV1 cell line lacks expression of ASS, and we have shown is auxotrophic for arginine.

Given our investigation of the role of autophagy in the cellular response of prostate cancer to

CWR-22Rv1 xenografts – ATG9A immunostaining



Control

PEG-ADI

PEG-ADI, we wished to develop a method to evaluate autophagy in xenografts for further in vivo characterization. Using the CWR22rV1 tumors from our initial in vivo experiment, we screened the tumors from the PEG-ADI treatment group for expression of a variety of autophagy-related proteins. Expression was evaluated using immunohistochemical techniques; we feel that this would provide us with the necessary method to further characterize the role of autophagy in tumor response. After screening a variety of antibodies, we noted that expression of ATG9A was increased in those tumors undergoing treatment with PEG-ADI (Figure 3). We are now performing in vitro studies to demonstrate that the ATG9A

Figure 3. Immunohistochemical expression of ATG9A in CWR22rV1 xenografts grown in athymic mice. Mice were treated with PBS (Control, left) or PEG-ADI (5 IU intraperitoneal injection, twice weekly). Increased expression of ATG9A was observed in those tumors from mice treated with PEG-ADI.

expression pattern is consistent with induction of autophagy and can be used as a marker in subsequent

in vivo studies.

The initial evaluation of biochemical signaling cascades altered following PEG-ADI treatment in CWR22rV1 demonstrated induction of AMP kinase and ERK1/2 (reference 1, Figure 4, attached in Appendix). AMP kinase is regulated by levels of AMP/ADP and is involved in initial responses to alteration in cellular metabolism. Both AMP kinase and ERK1/2 have been demonstrated to be involved in the regulation of autophagy, but not previously described in response to amino acid starvation (3, 4). We therefore sought to determine whether these kinases are central regulators of autophagy by examining CWR22rV1 cells following treatment with another inducer of autophagy, namely mTOR inhibition by rapamycin (5). We first demonstrated that rapamycin induces autophagy in these cells using our CWR22Rv1:GFP-LC3 cell line to evaluate for autophagosome assembly (data not shown).

We then examined the cells following treatment with rapamycin (2 μ M) for various time points. As expected, we noted rapid and potent inhibition of S6 kinase, a downstream partner in the mTOR signaling cascade. But interestingly, we observed an activation of AMP kinase, which is postulated to be upstream of mTOR (Figure 4). Therefore, using two different methods to induce autophagy (amino acid deprivation with PEG-ADI and mTOR inhibition with rapamycin), we note the induction of AMP kinase activity. Our future investigations will determine the role of AMP kinase in autophagy and cell death induced by PEG-ADI.

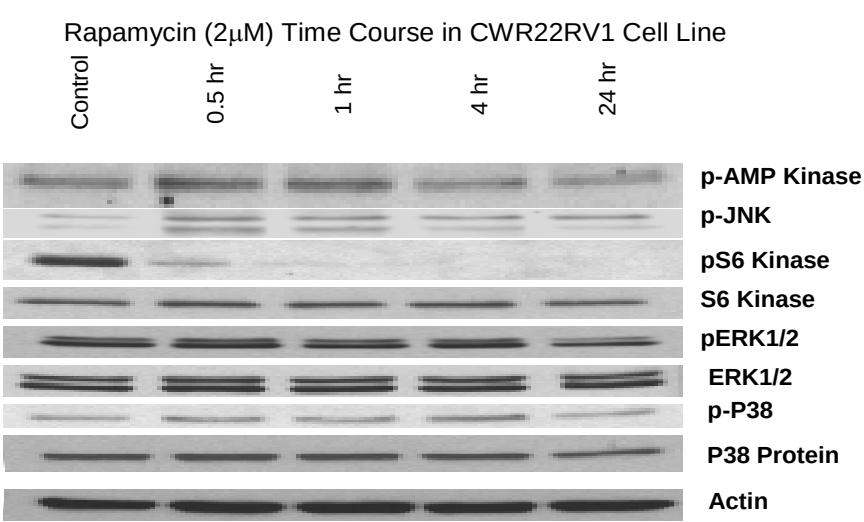


Figure 4. Time course of rapamycin (mTOR inhibition) in CWR22rV1 cells evaluated for protein expression of indicated kinases, including phosphorylated forms to evaluate for activation/inhibition.

Key Research Accomplishments

1. Characterization of prostate cancer cell lines and tumor specimens for ASS expression
2. Characterization of prostate cancer cell lines for cellular response to arginine deprivation
3. Creation of stable cell lines of PC-3 and CWR22rV1 expressing eGFP-LC3 for use in evaluation of the autophagy response
4. Development of a non-radioactive assay for arginine metabolism
5. Cloning of the ASS cDNA and development of a eukaryotic expression vector
6. Identification of ATG9A as an appropriate immunohistochemical marker of autophagy in xenograft specimens

Reportable Outcomes

Manuscripts:

1. Kim, R.H., J.M. Coates, T.L. Bowles, G.P. McNerney, J. Sutcliffe, J.U. Jung, R. Gandour-Edwards, F.Y.S. Chuang, **R.J. Bold**, and H-J. Kung. Arginine deiminase as a novel therapy for prostate cancer induces autophagy and caspase-independent apoptosis. Cancer Research, 69(2):700-708, 2009.

2. Kim, R.H., **R.J. Bold**, and H-J. Kung. ADI, autophagy and apoptosis: Metabolic stress as a therapeutic option for prostate cancer. *Autophagy*. 5(4):567-8, 2009.

Conclusion

In our first of a three year grant entitled “Metabolic stress induced by arginine deprivation induces autophagy cell death in prostate cancer”, we have made significant progress in the investigation of our central hypothesis. We have completed two of the four sub-aims for Aim 1 (We will demonstrate that in those prostate cancer cells lacking ASS for which arginine is an essential amino acid, metabolic stress induced by arginine deprivation achieved by ADI-PEG treatment induces autophagic cell death as a precursor to apoptosis via a DRAM-dependent pathway). We proposed to investigate DRAM as a mediator of autophagy and plan on initiating these studies in the coming year. We have completed one of the two subaims of Aim 2 () We will demonstrate that the induction of PEG-ADI induced autophagy in prostate cancer cells is a precursor to apoptosis; furthermore, these events sensitize cells to traditional chemotherapy-induced apoptosis). In the coming year, we plan to investigate the interaction of arginine deprivation with traditional chemotherapy. Our preliminary data in vivo (reference 1, Figure 3, attached in Appendix) suggests and interaction between these two therapeutic approaches, though we will further characterize in vitro.

As far as the financial conduct of the research, we are well within the budget without any significant deviations noted or anticipated.

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Research Article

Arginine Deiminase as a Novel Therapy for Prostate Cancer Induces Autophagy and Caspase-Independent Apoptosis

Randie H. Kim,¹ Jodi M. Coates,² Tawnya L. Bowles,³ Gregory P. McNerney,³ Julie Sutcliffe,⁴ Jae U. Jung,⁶ Regina Gandour-Edwards,⁶ Frank Y.S. Chuang,³ Richard J. Bold,² and Hsing-Jien Kung¹

Departments of ¹Biological Chemistry, ²Surgery (Division of Surgical Oncology), ³Biophysics (Center for Biophysics and Science Technology), ⁴Biomedical Engineering, and ⁵Pathology, University of California at Davis, Sacramento, California, and ⁶Department of Molecular Microbiology and Immunology, University of Southern California Keck Medical School, Los Angeles, California

Abstract

Arginine deprivation as an anticancer therapy has historically been met with limited success. The development of pegylated arginine deiminase (ADI-PEG20) has renewed interest in arginine deprivation for the treatment of some cancers. The efficacy of ADI-PEG20 is directly correlated with argininosuccinate synthetase (ASS) deficiency. CWR22Rv1 prostate cancer cells do not express ASS, the rate-limiting enzyme in arginine synthesis, and are susceptible to ADI-PEG20 *in vitro*. Interestingly, apoptosis by 0.3 μ g/mL ADI-PEG20 occurs 96 hours posttreatment and is caspase independent. The effect of ADI-PEG20 *in vivo* reveals reduced tumor activity by micropositron emission tomography as well as reduced tumor growth as a monotherapy and in combination with docetaxel against CWR22Rv1 mouse xenografts. In addition, we show autophagy is induced by single amino acid depletion by ADI-PEG20. Here, autophagy is an early event that is detected within 1 to 4 hours of 0.3 μ g/mL ADI-PEG20 treatment and is an initial protective response to ADI-PEG20 in CWR22Rv1 cells. Significantly, the inhibition of autophagy by chloroquine and Beclin1 siRNA knockdown enhances and accelerates ADI-PEG20-induced cell death. PC3 cells, which express reduced ASS, also undergo autophagy and are responsive to autophagy inhibition and ADI-PEG20 treatment. In contrast, LNCaP cells highly express ASS and are therefore resistant to both ADI-PEG20 and autophagic inhibition. These data point to an interrelationship among ASS deficiency, autophagy, and cell death by ADI-PEG20. Finally, a tissue microarray of 88 prostate tumor samples lacked expression of ASS, indicating ADI-PEG20 is a potential novel therapy for the treatment of prostate cancer. [Cancer Res 2009;69(2):700-8]

Introduction

The initial observations that various tumor cells are susceptible to arginine deprivation were made over 40 years ago, although appropriate therapeutic methods have hindered further development of this approach until recently. Arginine deiminase (ADI), an enzyme isolated from *Mycoplasma* (1, 2), degrades arginine into its citrulline precursor. In its native form, it is strongly antigenic with

a half-life of 5 hours (3). Conjugation to 20,000 mw polyethylene glycol (ADI-PEG20) decreases antigenicity as well as dramatically increases serum half-life, allowing weekly administration that reduces plasma arginine to undetectable levels (4, 5). Various tumor types (hepatocellular carcinomas, melanomas, mesotheliomas, renal cell carcinomas, pancreatic carcinomas) have been shown to lack expression of argininosuccinate synthetase (ASS; refs. 4, 6-8), a ubiquitous enzyme involved in the two-step synthesis of arginine from citrulline (9). Unable to synthesize their own arginine, ASS-deficient cells depend on relatively inefficient amino acid transporters (10). In the setting of ASS deficiency, ADI-PEG20 depletes intracellular arginine by reducing extracellular levels available for transmembrane uptake while unaffected cells with preserved ASS expression capable of endogenous arginine biosynthesis (11). Previous *in vitro* studies show the growth of prostate cancer PC3 cells is inhibited when arginine is eliminated from cell culture medium (12), indicating ADI-PEG20 may be an effective therapy for prostate cancers.

The antitumor effects of ADI-PEG20 elicit a G₁ cell cycle arrest with eventual apoptosis in a number of tumor cell lines (13). In addition, ADI-PEG20 is antiangiogenic, inhibiting migration and tube formation in HUVE cells (14) and neovascularization of neuroblastomas *in vivo* (15). However, other cellular effects of arginine starvation by ADI-PEG20 are still unknown.

Nutrient depletion triggers a process called macroautophagy (hereafter called autophagy), an evolutionary conserved eukaryotic process in which organelles and bulk proteins are turned over by lysosomal activity. Autophagy serves to provide ATP and other macromolecules as energy sources during metabolic stress (16, 17). The most distinctive feature of autophagy is the formation of the autophagosome, a double-membrane vesicle that fuses with lysosomes for hydrolytic cleavage of engulfed proteins and organelles. In mammalian cells, microtubule-associated protein 1 light chain 3 (LC3) is processed by lipid conjugation to phosphatidylethanolamine for insertion into the autophagosome membrane (18). Translocation and processing of an eGFP-LC3 fusion protein are often used as markers for autophagic activity.

Autophagy has recently gained much attention for its paradoxical roles in cell survival and cell death, particularly in the pathogenesis as well as the treatment of cancer (19, 20). Regulation of autophagy is highly complex with inputs from the cellular environment through the phosphatidylinositol-3-OH kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway (21), members of the Bcl2 family (22), p53 (23), and death-associated protein kinases (24). Not surprisingly, there is an intricate relationship between autophagy and apoptosis. Whether autophagy enables cells to survive or enhances their death is context-driven, depending on the type of stimuli, nutrient availability, organism

Requests for reprints: Hsing-Jien Kung, UC Davis Cancer Center, University of California - Davis Medical Center, Research III, Room 2400B, 4645 2nd Avenue, Sacramento, CA 95817. Phone: 916-734-1538; Fax: 916-734-2589; E-mail: hkung@ucdavis.edu and Richard J. Bold, University of California - Davis Medical Center, 4601 X Street, Sacramento, CA 95817. Phone: 916-734-5907; Fax: 916-733-5267; E-mail: richard.bold@ucdavis.ucdavis.edu.

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development, and apoptotic status. We hypothesize prostate cancer cells that are ASS deficient are sensitive to arginine deprivation by ADI-PEG20 and consequently, undergo autophagy as an initial survival response.

In this study, we show susceptibility of several prostate cancer cell lines to ADI-PEG20 correlates with the absence of ASS expression. Due to the lack of ASS, ADI-PEG20 induces a late caspase-independent cell death in CWR22Rv1 *in vitro*. Metabolic activity by micro positron emission tomography (microPET) imaging of CWR22Rv1 xenografts in nude mice was reduced by ADI-PEG20. Tumor growth was significantly inhibited by ADI-PEG20 alone as well as in combination with docetaxel. ADI-PEG20 also induces autophagy within hours of treatment. However, inhibition of autophagy prematurely leads to cell death by ADI-PEG20. With the success of ADI-PEG20 therapy for hepatocellular carcinomas and melanomas and our findings that prostate cancer specimens lack ASS expression, ADI-PEG20 can potentially be extended to clinical trials for prostate cancer. Moreover, combination with standard chemotherapies or autophagy-targeting drugs represents multipronged approaches to cancer therapy.

Materials and methods

Reagents. Recombinant ADI formulated with multiple linear 20,000 mw polyethylene glycol molecules (ADI-PEG20) was generously provided by DesignRx Pharmaceuticals, Inc. Specific enzyme activity was 7.4 IU/mg. Internal calibration of enzyme IC₅₀ was determined with each batch.

Cells and cell culture. All cell lines were cultured in RPMI 1640 [10% fetal bovine serum (FBS), 1% penicillin, streptomycin, glutamine]. LNCaP cells were cultured in serum-free, phenol-free medium before 10 nmol/L 5 α -Dihydrotestosterone (DHT; Sigma) treatment for 4, 24, and 48 h. PC3 cells were transiently transfected and CWR22Rv1 cells were stably transfected with eGFP-LC3 plasmid (UJ) using Effectene (Qiagen).

Reverse transcription-PCR and quantitative reverse transcription-PCR. Total RNA was isolated from cultured cells by TRIzol (Invitrogen) homogenization and reverse transcribed using Moloney murine leukemia virus (Invitrogen). One hundred nanograms of cDNA were PCR amplified as described previously (25). Primers: ASS (F) 5'-GACGCTATGTCAGCAAG-3' and (R) 5'-TTGCTTTGCGTACTCCATCAG-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH; F) 5'-ACCACAGTCCATGCCATCAC-3' and (R) 5'-TCCACCACCCTGTTGCTGTA-3'. Total RNA from primary prostate tissues was reverse transcribed using SuperScriptIII (Invitrogen). One hundred nanograms of cDNA were amplified by iQ5 iCycler thermal cycler (Bio-Rad) and monitored by SYBRGreen (Invitrogen) for real-time PCR. Threshold cycle values were normalized against actin and analyzed using QGene software. Primers were as follows: ASS (F) 5'-TGAAATTGCTGAGCTGTG-3' and (R) 5'-ATGTACACCTGCCCCCTTGA-3'; Actin (F) 5'-TCCTTAATGTCACGACGATT-3' and (R) 5'-GAGCGCGCTACAGCTT-3'.

Immunoblotting. Cellular lysates were resolved on SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were incubated with primary antibody followed by species-specific horseradish peroxidase secondary antibody. Immunoreactive material was detected by chemiluminescence (Pierce Laboratories). Antibodies were as follows: actin (Santa Cruz Biotechnology), ASS (BD Biosciences), caspase-3 (Biosource), GAPDH (Chemicon), tubulin (Sigma), Beclin1, phos-

pho-AMP kinase (Thr172), phospho-mTOR (Ser2481), phospho-S6 kinase (Thr389), phospho-S6 (Ser235/236), LC3, extracellular signal-regulated kinase (ERK1/2), and phospho-ERK1/2 (Cell Signaling).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cytotoxicity assay. RWPE-1, LNCaP, PC3, and CWR22Rv1 cells were seeded in 96-well plates and treated with serial dilutions of ADI-PEG20. After 6 d, thiazolyl blue tetrazolium bromide (MTT; Sigma) was added for a final concentration of 0.5 mg/mL. PC3 cells were treated for 3 d in 2% FBS. Formazan crystals were solubilized by 10% SDS. The IC₅₀ is the drug concentration at which 50% of cell growth is inhibited.

Fluorescence-activated cell sorting analysis for sub-G₁ DNA fragmentation. CWR22Rv1 cells were treated with 0.3 μ M ADI-PEG20, 100 nmol/L paclitaxel (Sigma), or pretreated with 50 μ M/L z-VAD-fmk (MBL International). Cells were analyzed by flow cytometry as described previously (8).

Active caspase-3 ELISA. CWR22Rv1 cells were seeded in 6-well plates and treated with 100 nmol/L paclitaxel or 0.3 μ M ADI-PEG20 for 24 h. Treatment groups were compared with cells pretreated with 50 μ M/L z-VAD-fmk for 2 h before assaying for activated caspase-3 by ELISA (R&D Systems).

MicroPET imaging. Nude mice with CWR22Rv1 s.c. xenografts were injected via tail vein with 120 mCi of ¹⁸F-FDG and imaged by PET as described previously (26) before and after 5 IU ADI-PEG20 treatment of 4 or 24 h. Standard uptake values (SUV) were computed by dividing the activity concentration in each voxel by the injected dose and multiplying by animal weight. Absolute uptake values of posttreatment images were normalized to pretreatment images before analysis.

Xenograft efficacy studies. For tumorigenesis, 1×10^6 CWR22Rv1 cells were injected s.c. into the bilateral flanks of male athymic BALB/c mice (Harlan Sprague-Dawley, Inc.). Mice received weekly 0.5 mL i.p. injections of sterile PBS ($n = 4$), 10 mg/kg docetaxel ($n = 4$), 5 IU (225 μ g/mL) ADI-PEG20 ($n = 4$), or both 10 mg/kg docetaxel and 5 IU ADI-PEG20 (1 mL total volume; $n = 4$). Tumor dimensions were measured twice weekly. Tumor volumes were calculated by $V = 0.5236 (L \times W^2)$, L = length, W = width.

Fluorescence microscopy for LC3. CWR22Rv1 and PC3 cells overexpressing eGFP-LC3 were seeded on poly-lysine-coated coverslips. Cells were treated with 0.3 μ M ADI-PEG20 for 4 or 24 h or 2 μ M/L rapamycin for 4 h. Cells were fixed, mounted using SlowFade with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen), and examined under a $\times 60$ lens on an Olympus BX61 motorized reflected fluorescence microscope with an AMCA filter (excitation, 350 nm; emission, 460 nm) for DAPI and FITC filter (excitation, 480 nm; emission, 535 nm) for eGFP-LC3 using SlideBook4.1 software (Intelligent Imaging Innovations).

For live cell imaging. CWR22Rv1 cells overexpressing eGFP-LC3 were plated on 35 mm #1 glass bottom dishes (WillCo Wells), treated with 0.3 μ M ADI-PEG20, and imaged with an IX-71 inverted microscope with a $\times 100$ 1.40 NA oil objective (Olympus) and ASI 400 air stream incubator (NEVTEK) set to 37°C. Images were acquired using a spinning disc system.

Inhibition of autophagy. CWR22Rv1 cells were treated with 25 μ M/L chloroquine (Sigma), 0.1 μ M ADI-PEG20, or both for 24, 48, 72, and 96 h. LNCaP cells were treated as above except with 0.3 μ M ADI-PEG20. Cells were analyzed by fluorescence-activated cell sorting (FACS) analysis as described previously.

CWR22Rv1 cells were seeded in 6-well plates then transiently transfected with 100 pmol eGFP siRNA (Ambion) or Beclin1 siRNA ON-TARGETplus SMARTpool (Dharmacon) using DharmaFECT

reagent (Dharmacon). Cells were treated with 0.3 $\mu\text{g}/\text{mL}$ ADI-PEG20 for 24 or 48 h the following day and analyzed by FACS analysis as described previously.

PC3 cells were treated with 0.3 $\mu\text{g}/\text{mL}$ ADI-PEG20, 5 $\mu\text{g}/\text{mL}$ ADI-PEG20, 1 mmol/L 3-methyladenine (3-MA; Sigma), or both for 24, 48, and 72 h and analyzed by MTT as described previously.

ASS immunohistochemistry. Formalin-fixed, paraffin-embedded archival material from 88 prostate tumors and 59 normal prostate samples were obtained. Tumors represent a range of Gleason grades (3+3 = 6 to 4+5 = 9). H&E-stained sections were made from

each block to define representative tumor regions, and a tumor microarray (TMA) was constructed. TMA paraffin blocks were sectioned at 4 μm and transferred to glass slides. Immunohistochemistry was performed using α -ASS monoclonal mouse antibody (DesignRx Pharmaceuticals) at 2.2 $\mu\text{g}/\text{mL}$. Normal liver was used as a positive control. Omission of primary antibody was used as negative control. Sections were counterstained with Gill's hematoxylin and fixed. Slides were independently examined by a board certified anatomic pathologist (RGE) three times and scored by percentage of cells stained.

Results

Sensitivity to ADI-PEG20 correlates with ASS expression. ASS expression in three commonly cultured prostate carcinoma cell lines (LNCaP, PC3, CWR22Rv1) was evaluated for mRNA and protein levels. LNCaP is androgen dependent, whereas PC3 and CWR22Rv1 are androgen independent. The normal immortalized cell line RWPE-1 was used to evaluate ASS expression in noncancerous prostate cells. All cell lines expressed ASS mRNA determined by reverse transcription-PCR (RT-PCR) except CWR22Rv1 (Fig. 1A). Quantitative real-time PCR of ASS mRNA in the prostate cancer cell lines revealed that, relative to CWR22Rv1, LNCaP and PC3 expressed ASS transcript 6.7 and 1.4 times greater, respectively. Western blot analysis showed CWR22Rv1 did not express ASS protein; in contrast, PC3 expressed moderate levels, whereas LNCaP and RWPE-1 expressed high levels of ASS (Fig. 1B). Disparity between ASS mRNA and protein levels is potentially attributed to nonproductive, alternatively spliced transcripts or pseudogenes.⁷ The relationship between androgen status and ASS expression was further examined by treating LNCaP cells with 10 nmol/L DHT (Fig. 1C), revealing androgens do not regulate ASS expression.

To evaluate the effect of ADI-PEG20 on prostate carcinoma, the previously described cell lines were treated with ADI-PEG20 over a broad dose range and assayed for cytotoxicity with MTT. CWR22Rv1 was the most sensitive to ADI-PEG20 with an IC_{50} of 0.3 $\mu\text{g}/\text{mL}$. PC3 was moderately sensitive to ADI-PEG20, whereas LNCaP and RWPE-1 were not responsive to ADI-PEG20 (Fig. 1D). Taken together, these data confirm that ASS protein levels inversely correlate with sensitivity to ADI-PEG20. CWR22Rv1 was subsequently chosen as the model cell line for future experiments.

ADI-PEG20 induces caspase-independent apoptosis in CWR22Rv1 *in vitro*. To study whether the reduced viability of CWR22Rv1 upon ADI-PEG20 treatment is due to cell growth arrest, apoptosis, or both, we subjected treated and untreated cells to FACS analysis. The sub-G₁ DNA content was used as an indicator of apoptosis induced by ADI-PEG20. CWR22Rv1 cells were treated with 0.3 $\mu\text{g}/\text{mL}$ ADI-PEG20 for 4, 24, 48, 72, and 96 hours. Apoptosis was not induced until 4 days posttreatment, when $\sim 30\%$ of cells had undergone apoptosis (Fig. 2A). Although DNA fragmentation is considered the defining end point in apoptosis, caspase cleavage is an early marker for classic apoptosis. Interestingly, cleavage of caspase-3 into its activated 17 kDa fragment was undetected after ADI-PEG20 (Fig. 2B).

Caspase-independent cell death was further investigated with z-VAD-fmk, a pan-caspase inhibitor. Caspase inhibition was confirmed with caspase-3 ELISA (Fig. 2C). z-VAD-fmk led to a 50%

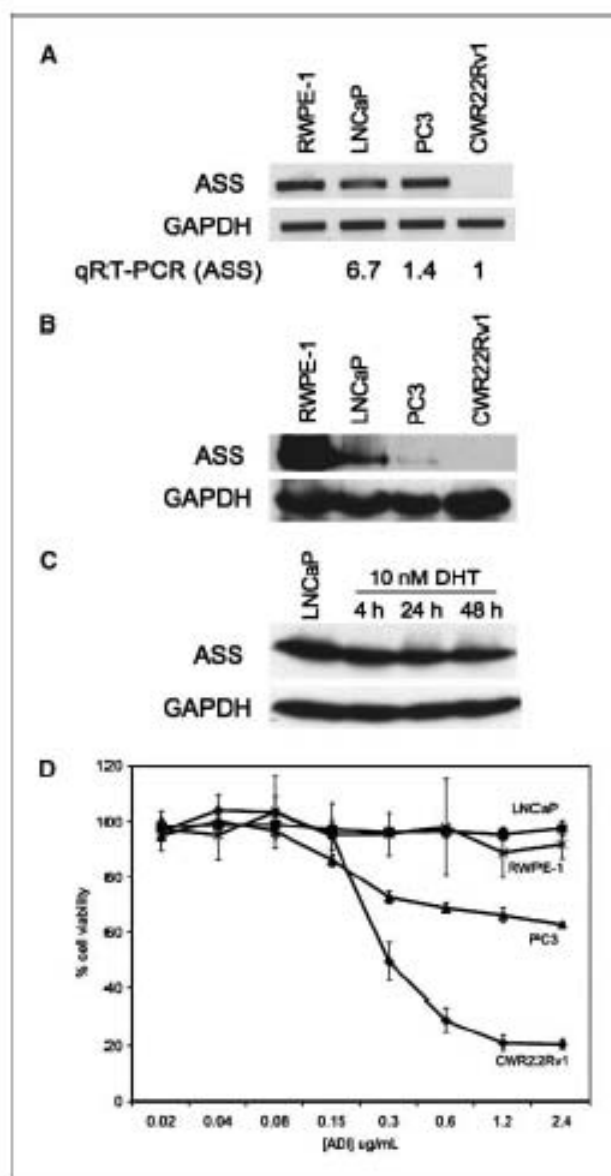


Figure 1. Prostate cancer cell lines profiled for ASS expression and ADI-PEG20 sensitivity. RWPE-1, LNCaP, PC3, and CWR22Rv1 were examined for ASS mRNA by RT-PCR (A) and ASS protein by immunoblotting (B). C, Immunoblot for 10 nmol/L DHT time course of LNCaP against α -ASS. D, Cell lines were treated by ADI-PEG20 at 0.02, 0.04, 0.08, 0.15, 0.3, 0.6, 1.2, and 2.4 $\mu\text{g}/\text{mL}$ for 3 (PC3) or 6 d before MTT assay. Points, mean; bars, SD.

⁷ Pei-Jer Chen, personal communication.

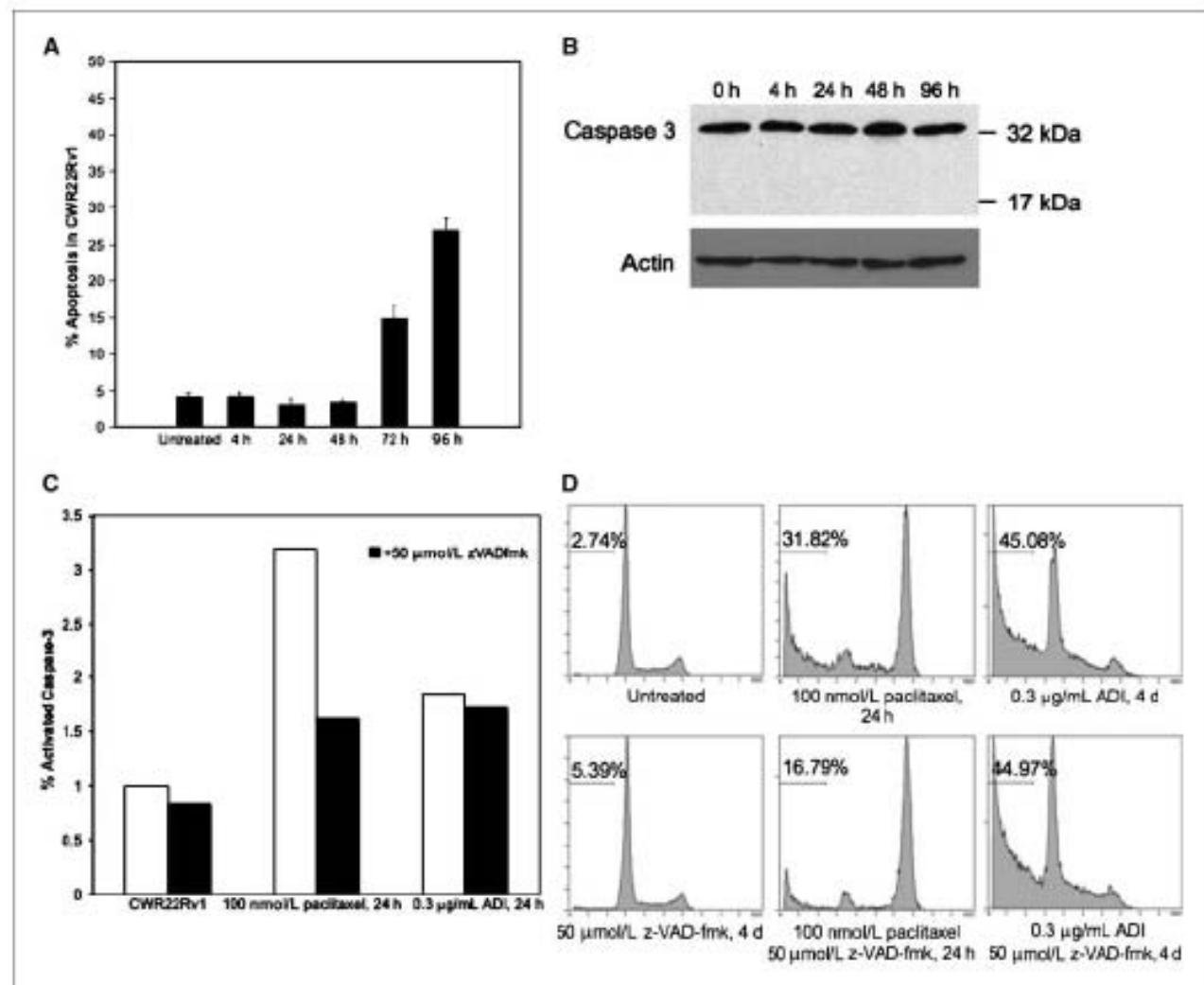


Figure 2. ADI-PEG20 induces caspase-independent apoptosis in CWR22Rv1. **A**, 0.3 μg/mL ADI-PEG20 time course of CWR22Rv1 before FACS analysis for sub-G₁ DNA content. Columns, mean; bars, SE. **B**, Immunoblot for 0.3 μg/mL ADI-PEG20 time course of CWR22Rv1. α-Caspase-3 detects the 32 kDa proform and the activated 17 kDa cleavage product. **C**, CWR22Rv1 cells were treated with vehicle (untreated), 100 nmol/L paclitaxel, or 0.3 μg/mL ADI-PEG20 for 24 h and compared with 2 h pretreatment with 50 μmol/L z-VAD-fmk before caspase-3 ELISA. Values were normalized to vehicle. **D**, CWR22Rv1 cells were treated with 50 μmol/L z-VAD-fmk, 0.3 μg/mL ADI-PEG20, ADI-PEG20+z-VAD-fmk for 96 h, and 100 nmol/L paclitaxel, paclitaxel+z-VAD-fmk for 24 h before FACS analysis for sub-G₁ DNA content.

reduction of activated caspase-3 levels in cells treated with paclitaxel, a standard chemotherapy for advanced and metastatic prostate cancer. However, ADI-PEG20 did not significantly alter active caspase-3 levels. Although z-VAD-fmk attenuated apoptosis in cells treated with paclitaxel by ~50%, it did not affect the fraction of apoptotic cells after ADI-PEG20 (Fig. 2D). These data suggest that cell death mediated by ADI-PEG20 is independent of caspase-mediated pathways.

ADI-PEG20 decreases global tumor metabolic activity. The immediate effect of ADI-PEG20 *in vivo* was examined using PET. Global tumor metabolism of glucose consumption was monitored by ¹⁸F-fluorodeoxyglucose (¹⁸F-FDG) in CWR22Rv1 mouse xenografts. MicroPET scans were performed before and after ADI-PEG20 treatment of 4 or 24 hours. ¹⁸F-FDG uptake in CWR22Rv1 tumors (*μ*g/min) did not change after 4 hours of treatment. In

contrast, ¹⁸F-FDG uptake was decreased after 24 hours of ADI-PEG20. Tumor SUV decreased 30% after treatment (0.00086 versus 0.0006), indicating reduced metabolic activity (Fig. 3A).

ADI-PEG20 retards CWR22Rv1 tumor growth *in vivo* and synergizes with taxane. To determine the long term effects of ADI-PEG20 *in vivo*, nude athymic mice with s.c. CWR22Rv1 xenografts were injected i.p. with control PBS or 5 IU ADI-PEG20 weekly. Tumors from ADI-PEG20 mice were significantly smaller than tumors from control mice (157.6 mm³ versus 1,108.99 mm³) at 13 days after initiation of treatment when control mice were euthanized. The effects of ADI-PEG20 were compared with the current standard of care for hormone refractory prostate cancer patients, docetaxel alone (27), and docetaxel in combination. Docetaxel mice (10 mg/kg) had tumors that were smaller but not statistically significant from control mice. However, the

combination of ADI-PEG20 and docetaxel had a synergistic effect on tumor growth inhibition. Tumors from ADI-PEG20 mice reached an average of 910 mm³ at the end of the study whereas tumors from ADI-PEG20/docetaxel-treated mice were ~75% smaller (Fig. 3B).

ADI-PEG20 induces autophagy in prostate cancer cells. Arginine degradation by ADI-PEG20 causes metabolic stress to autotrophic cells. Nutrient starvation such as complete amino acid deprivation is a known inducer of autophagy (28). To determine whether single amino acid deprivation is sufficient to trigger autophagy, CWR22Rv1 cells stably expressing eGFP-LC3 were examined under fluorescence microscopy. Under normal conditions, LC3-I is uniformly distributed throughout the nucleus and cytoplasm. During autophagy, LC3-I is processed into LC3-II and translocates into autophagosome membranes, appearing as bright punctae (29). LC3-II localization was seen in fixed CWR22Rv1 cells after 4 and 24 hours of 0.3 µg/mL ADI-PEG20 treatment. Rapamycin, an inhibitor of mTOR, was used as a positive control (Fig. 4A, top). Live cell imaging of CWR22Rv1 cells revealed rapid and intense autophagosome formation after only 90 minutes of ADI-PEG20 (Fig. 4A, bottom). Rapamycin or ADI-PEG20 significantly increased the number of cells undergoing autophagy to 15%

(Fig. 4A). The LC3-II fragment appeared as early as 30 minutes of ADI-PEG20 and persisted after 24 hours of arginine deprivation. Increase in total autophagic flux was confirmed with chloroquine (30), an autophagy inhibitor that disrupts lysosomal function (Fig. 4B) and prevents completion of autophagy, resulting in an accumulation of LC3-II. In addition, potential off-target effects of chloroquine did not lead to caspase-3 cleavage.

Molecular pathways accompanying the induction of autophagy were also investigated. A major nutrient-sensing pathway involves AMPK/TSC/mTOR/S6K. During nutrient starvation, ATP level decreases and AMP level increases, resulting in activation and phosphorylation of AMPK. ADI-PEG20 immediately increased phospho-AMPK levels (Fig. 4C). This should lead to inactivation and decreased phosphorylation of mTOR kinase through the inhibition of TSC complex by AMPK-induced phosphorylation. Decreased phosphorylation of mTOR was evident soon after ADI-PEG20 treatment (Fig. 4C). A downstream mTOR effector, S6K, was inactivated at a later stage (>24 hours) as shown by its own decreased phosphorylation and the decreased phosphorylation of its substrate S6. Transient increase of S6K activity was observed at early ADI-PEG20 time points. The exact mechanism of this phenomenon is unclear but is likely due to feedback of this kinase as reported by others (31). AMPK activation and mTOR down-modulation are compatible with their roles of major autophagy regulators. We also surveyed other kinase pathways relevant to autophagy. ERK1/2 phosphorylation was evident within 30 minutes of ADI-PEG20 treatment, which increased in a time-dependent manner (Fig. 4C). ERK1/2 activation has been shown previously to contribute to autophagy induced pro-survival function (32).

Autophagy delays and protects against ADI-PEG20-induced cell death. The paradoxical relationship between autophagy and apoptosis necessitates determination of the causal nature between these two fundamental biological processes after arginine deprivation. Temporally, autophagy precedes apoptosis; thus, inhibition of autophagy may modulate the onset of apoptosis.

Chemical inhibition of autophagy with chloroquine accelerated and enhanced ADI-PEG20-induced cell death in CWR22Rv1 (Fig. 5A). By 48 hours, 27% of ADI-PEG20 + chloroquine cells were apoptotic compared with 11% and 6% of cells undergoing apoptosis by chloroquine alone and ADI-PEG20 alone, respectively. Chloroquine further increased ADI-PEG20-induced cell death to 60% after 72 hours. By 96 hours, the effect of chloroquine was abrogated, possibly due to its metabolism. Similarly, siRNA knockdown of Beclin1, essential for autophagosome nucleation (21), also increased the rate of cell death after ADI-PEG20 treatment (Fig. 5B). Almost 60% of cells had undergone apoptosis if Beclin1 was knocked down before 48 hours of ADI-PEG20, whereas ADI-PEG20 alone only led to apoptosis in 30% of cells. In contrast, ADI-PEG20, chloroquine, and the combination of ADI-PEG20 and chloroquine had no effect on apoptosis at all time points in the ASS expressing LNCaP cells (Fig. 5C). To complete the characterization of the relationship of ASS expression and sensitivity to ADI-PEG20, we examined cellular response in PC3, a cell line with low ASS levels. Higher doses of ADI-PEG20 (5 µg/mL) were required to arrest cell growth completely compared with CWR22Rv1, although lower doses (0.3 µg/mL) induced autophagy (Fig. 5D). Inhibiting autophagy with 3-MA greatly reduced cell viability following treatment with low dose ADI-PEG20 (Fig. 5D). Therefore, ASS protein level correlates with cellular response to ADI-PEG20, including the early induction of autophagy before the late onset of apoptosis.

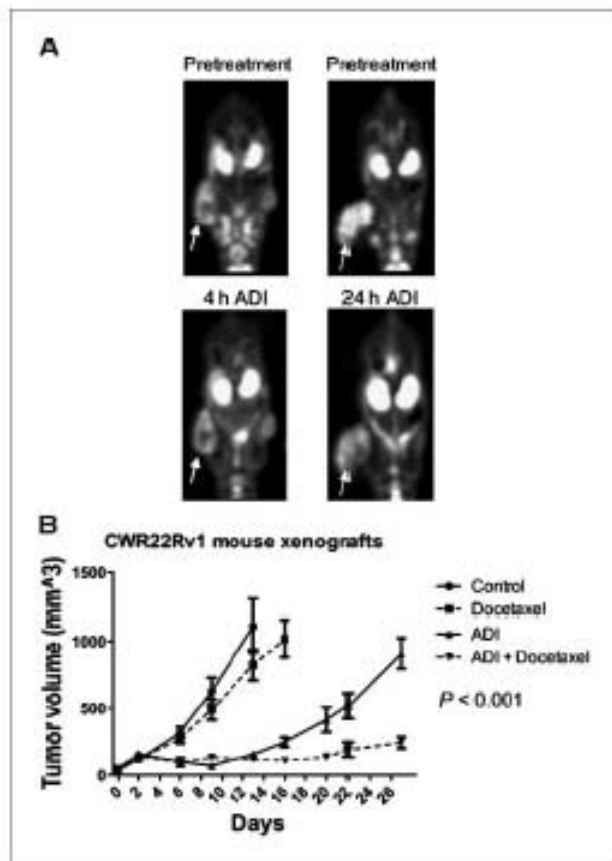
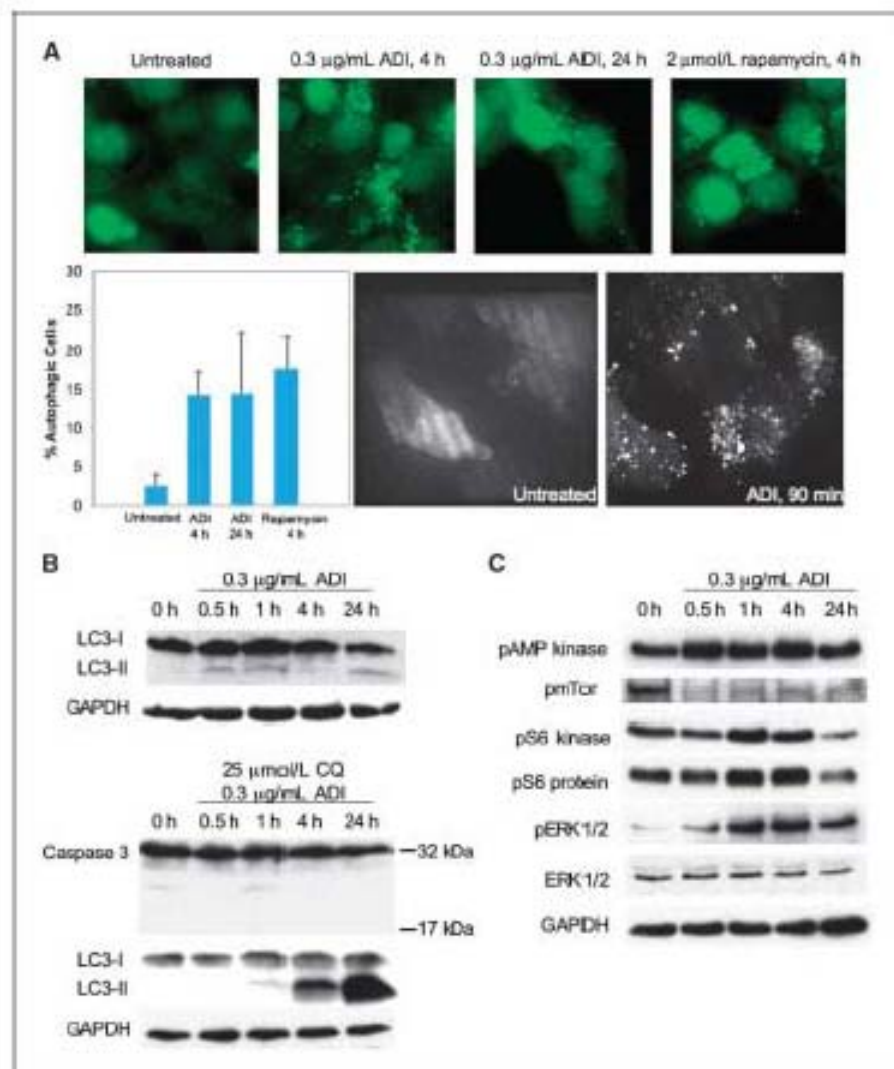


Figure 3. ADI-PEG20 is an effective agent *in vivo*. **A**, mice with CWR22Rv1 xenografts were imaged by PET using ¹⁸F-FDG before and after treatment with 5 IU ADI-PEG20 for 4 or 24 h. **B**, mice with CWR22Rv1 xenografts were treated with PBS vehicle, 10 mg/kg docetaxel, 5 IU ADI-PEG20, or 5 IU ADI-PEG20+10 mg/kg docetaxel weekly. Tumor volumes are reported as mean ± SE.

Figure 4. ADI-PEG20 induces autophagy in CWR22Rv1. **A**, CWR22Rv1 cells overexpressing eGFP-LC3 were treated with 0.3 μ M ADI-PEG20 for 1.5, 4, and 24 h, or 2 μ M rapamycin for 4 h. Punctate represent autophagosome formation. Autophagic cells were quantified from random image fields totaling 200 cells and reported as mean $\pm$ SE. **B**, Immunoblot for 0.3 μ M ADI-PEG20 time course of CWR22Rv1. α -LC3 detects LC3-I and LC3-II. Autophagic flux was confirmed by coadministering 25 μ M chloroquine with 0.3 μ M ADI-PEG20. α -Caspase-3 detects the 32 kDa proform and the activated 17 kDa cleavage product. **C**, Immunoblot for 0.3 μ M ADI-PEG20 time course of CWR22Rv1 using α -phospho-AMP kinase, α -phospho-mTOR, α -phospho-S6 kinase, α -phospho-S6 protein, α -phospho-ERK1/2, and α -ERK1/2. Loading control for phospho-mTOR was verified using α -tubulin (data not shown).



ASS expression in prostate cancer tissue. The above results suggest arginine deprivation by ADI-PEG20 may offer a new treatment strategy for prostate cancers in which ASS expression is low. A key question that follows is whether the absence of ASS expression is generalizable among diverse human prostate cancer specimens. We therefore examined ASS expression by immunohistochemistry in our prostate tissue microarray. Of the 88 human prostate tumors, none showed any detectable ASS staining. Strong cytoplasmic ASS staining was observed, indicated by closed arrows, in the luminal cells of benign prostate glands (Fig. 6A) and normal prostate tissue (Fig. 6B, left). However, no ASS reactivity was detected in prostate cancer glands (Fig. 6A, open arrows) or tissue (Fig. 6B, right). Among 59 samples of normal prostate tissue, 27% expressed ASS to some degree. Of the 16 samples showing ASS expression, 2 were found to have ASS in >75% of the cells, whereas the remaining 14 showed expression in <25% of the cells. In addition, ASS mRNA expression was evaluated in six primary prostate tumor tissues and two primary benign prostatic hyperplasia tissues. ASS mRNA was almost absent in specimen

108 and reduced in all other samples (Fig. 6C). The differential expression of ASS is in contrast to hepatocytes, which heavily depend on ASS function for the urea cycle, and uniformly stained for cytoplasmic ASS protein (Fig. 6D).

Discussion

In this report, we showed ADI-PEG20 can effectively induce cell death in prostate cancer cells with low or absent ASS expression. It also sensitizes cells to treatment with docetaxel, an accepted chemotherapy in prostate cancer, or chloroquine, an inhibitor of autophagy. These results are likely to be generally applicable to other prostate cancer cells because virtually all prostate cancer specimens examined in this report as well as that by Clark and colleagues (12) expressed undetectable levels of ASS. By depletion of arginine, ADI-PEG20 causes metabolic stress on auxotrophic cells, complementing conventional therapies largely based on genotoxic stress. Although arginine deprivation therapy based on bovine arginase has seen limited applications clinically ADI-PEG20

has 1,000-fold greater affinity for arginine (33) with fewer side effects. Our work described here thus offers a new treatment option for prostate cancer. In addition, we uncover novel cellular responses of arginine depletion, including autophagy and caspase-independent cell death.

The delayed onset of apoptosis suggests the possibility of compensation mechanisms after arginine depletion. Here, we present evidence for the first time that single amino acid starvation through arginine degradation by ADI-PEG20 is sufficient to trigger autophagy in prostate cancer cells. LC3 translocation and cleavage occur within hours of ADI-PEG20 treatment, indicating that autophagy is an early response. AMPK senses cellular AMP/ATP ratio, and in its phosphorylated form, signals the lack of nutrients in the environment to the mTOR complex via TSC2 (34). Inhibition of mTOR leads to suppression of S6K activity. Consistent with our findings, Feun and colleagues (35) have reported the effects of ADI-PEG20 on mTOR signaling, which include dephosphorylation of mTOR downstream effectors S6K and 4E-BP and increased phosphorylation of AMPK in ASS-negative melanoma cell lines. This chain of events has been shown to promote autophagy (36).

There are various signaling cascades that regulate mTOR/S6K including the PI3K (class I)/Akt pathway; inhibition of which has been shown to induce autophagy in malignant gliomas (32, 37). Although we did not specifically examine the activation of the PI3K (class I)/Akt pathway, ADI-PEG20 inhibited mTOR events associated with a rapid activation of AMPK, suggesting this mechanism in arginine deprivation-induced autophagy. Furthermore, we observed ADI-PEG20-induced ERK1/2 activation, which has been shown to regulate autophagy under a variety of stimuli (32, 38).

What is the biological function of ADI-PEG20 induced autophagy? Autophagy can be prosurvival or prodeath, depending on cellular context and duration of treatment. To study whether ADI-PEG20 induced autophagy contributes to or attenuates cell death, we chose to block ADI-PEG20 induced autophagy with the inhibitor chloroquine, which inhibits late stage autophagy by alkalinizing lysosomes and disrupting the autophagolysosome (39). Because chloroquine itself may have functions other than inactivating lysosomes (40), we also used siRNA targeting an essential component of autophagy, Beclin1, a component of the class III PI3 kinase complex that nucleates autophagosomes (29).

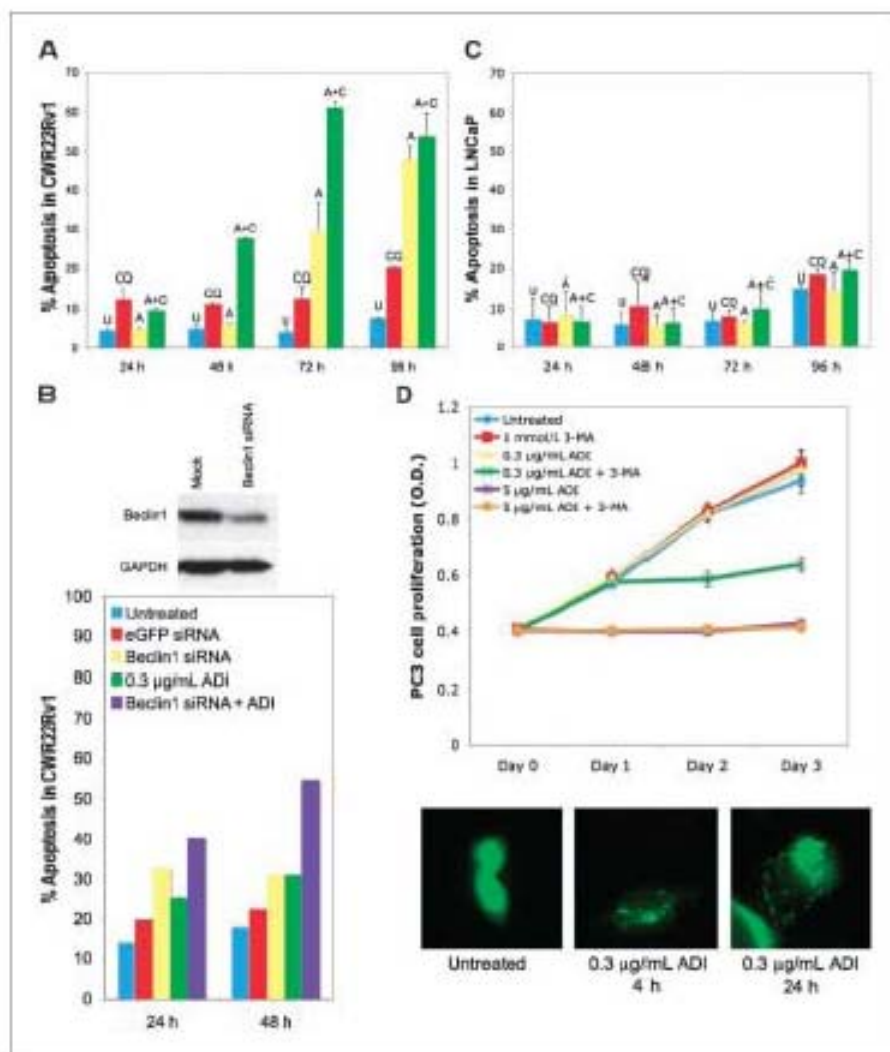
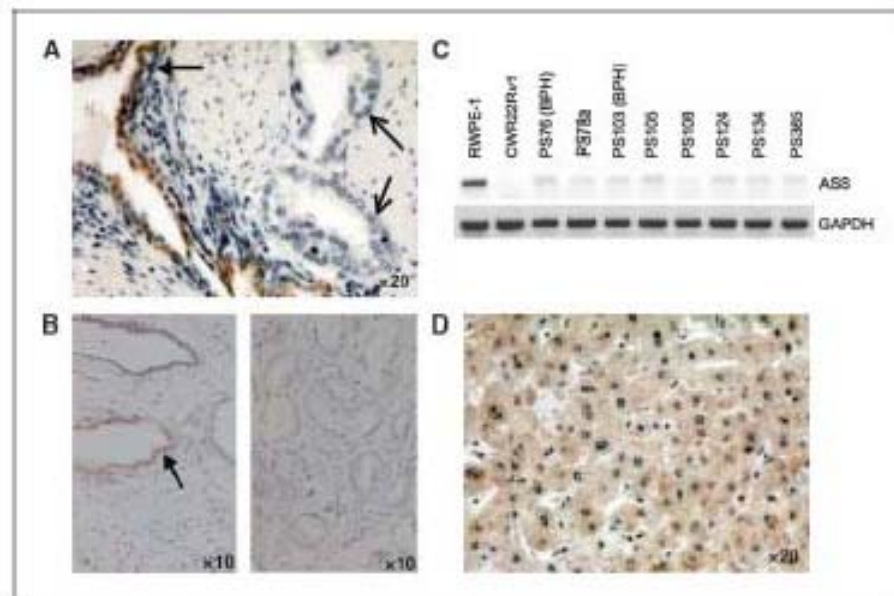


Figure 5. Inhibition of autophagy accelerates and enhances ADI-PEG20-induced cell death. **A**, time course of CWR22Rv1 cells treated with vehicle (untreated), 25 µmol/L CQ, 0.1 µg/mL ADI-PEG20, or ADI-PEG20 + CQ before FACS analysis for sub-G₁ content.

Columns, mean; bars, SE. **B**, Immunoblot for CWR22Rv1 cells transfected with mock or 100 pmol Beclin1 siRNA to assess knockdown. CWR22Rv1 cells were treated with vehicle (untreated), 0.3 µg/mL ADI-PEG20, 100 pmol eGFP siRNA, 100 pmol Beclin1 siRNA, or Beclin1 siRNA + ADI-PEG20 for 24 and 48 h before FACS analysis for sub-G₁ content.

C, LNCaP cells were treated and analyzed as described in **A** with 0.3 µg/mL ADI-PEG20. **D**, growth of PC3 cells were treated with vehicle (untreated), 0.3 µg/mL ADI-PEG20, 1 mmol/L 3-MA, 0.3 µg/mL ADI-PEG20 + 1 mmol/L 3-MA, 5 µg/mL ADI-PEG20, or 5 µg/mL ADI-PEG20 + 1 mmol/L 3-MA by MTT assay. Points, mean; bars, SD. PC3 cells overexpressing eGFP-LC3 were treated with 0.3 µg/mL ADI-PEG20 for 4 or 24 h. Punctae represent autophagosome formation. U, untreated; CQ, chloroquine; A, ADI-PEG20.

Figure 6. ASS expression in prostate tissue. **A**, prostate cancer tissue with ASS(+) benign glands (closed arrows) and ASS(−) cancerous glands (open arrows) by immunohistochemistry. **B**, left, normal prostate tissue. Closed arrows, luminal ASS staining. Right, prostate cancer tissue with no ASS reactivity. **C**, mRNA from primary prostate tissue was examined for ASS expression by RT-PCR. **D**, normal liver as a positive control for cytoplasmic ASS staining. BPH, benign prostatic hyperplasia.



Our data show inhibition of early stage autophagy by chloroquine or Beclin1 knockdown accelerates and enhances cell death after ADI-PEG20, strongly suggesting ADI-PEG20-induced autophagy triggers a protective response during early stages of treatment. At present, we cannot rule out that prolonged ADI-PEG20 treatment (>96 hours) may trigger autophagic cell death (programmed cell death type II), which is usually caspase-independent. In our study, we found chloroquine itself had little effect on the cell killing of CWR22Rv1, unless ADI-PEG20 is present and autophagy is induced. In addition, coadministration of chloroquine with ADI-PEG20 did not activate caspase-3. This again suggests that the major effect of chloroquine is to block autophagy, enhancing the underlying mechanism of caspase-independent apoptosis. Consistent with this result, PC3 cells with reduced ASS levels also underwent autophagy after ADI-PEG20 treatment. The inhibition of autophagy with 3-MA significantly reduced cell proliferation in the presence of ADI-PEG20. Both chloroquine and ADI-PEG20 have no effect on LNCaP cells, which express ASS. Interestingly ASS-positive hepatocellular carcinomas resistant to ADI-PEG20 responded to arginine deprivation by pegylated recombinant arginase (41), providing a potential alternative to ADI-PEG20-resistant tumors and cell lines such as LNCaP.

In cancer, an autophagy paradox has emerged in which survival and death are context specific, particularly due to complex interactions between autophagic and apoptotic pathways. Accordingly, cancer therapies have been reported to have opposing effects on cell death. Photodynamic therapy promotes autophagic cell death in apoptosis-deficient cancer cells (42), whereas sulforaphane-induced autophagy in PC3 and LNCaP is protective (43). Furthermore, manipulation of autophagy can sensitize tumor cells to subsequent treatments. Induction of autophagy by an mTOR inhibitor increased prostate cancer cell susceptibility to irradiation (44). Conversely, chloroquine is a highly promising autophagy inhibitor for clinical use. Although it is extensively used to treat malaria (20), its uses against cancer are only recently emerging. In a myc-induced lymphoma model, autophagic

inhibition by chloroquine enhanced the ability of alkylating agents to suppress tumor growth (45). This underscores the importance of autophagy to fundamental cell processes and its ability to modulate the effect of chemotherapies across a wide variety of cancers.

The absence of ASS as a biomarker for ADI-PEG20 efficacy has previously been established in hepatoma and melanoma cell lines. Phase I/II clinical trials with ADI-PEG20 led to a 47% response rate in patients with unresectable hepatocellular carcinomas and a 25% response rate in metastatic melanoma patients (46, 47). In this study, we show ADI-PEG20 can be effective against prostate cancer. ASS expression can be determined by immunohistochemistry and potentially be used as a clinical indicator for ADI-PEG20 use. The absence of ASS protein in all examined prostate tumor samples makes ADI-PEG20 a promising therapeutic avenue to follow. The combination of ADI-PEG20, which induces caspase-independent apoptosis, and taxanes, which are caspase-dependent, is more effective than monotherapy. This concept of synergistic interaction between cancer therapies is an active area of research. In particular, combining therapies that target different mechanisms of cell death may increase efficacy beyond either agent alone. Furthermore, the increase of advanced imaging for tumor assessment and staging may allow clinical monitoring of tumor responsiveness to ADI-PEG20 by PET. Finally, arginine deprivation by ADI-PEG20 induces autophagy as a protective mechanism. Coadministration with an autophagic inhibitor such as chloroquine can potentially enhance cell death in prostate tumors. The intricate link between autophagy and apoptosis points to autophagy as an additional target for anticancer treatments. Thus, ADI-PEG20 is a novel prostate cancer therapy whose mechanism of action can be complemented by other chemotherapies to maximize cell death.

Disclosure of Potential Conflicts of Interest

RJ, Bold: commercial research grant, DesignRx Pharmaceuticals. The other authors disclosed no potential conflicts of interest.

Acknowledgments

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